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Homocysteine – from disease biomarker to disease prevention

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We have reviewed the literature and have identified more than 100 diseases or conditions that are associated with raised concentrations of plasma total homocysteine. The commonest associations are with cardiovascular diseases and diseases of the central nervous system, but a large number of developmental and age-related conditions are also associated. Few other disease biomarkers have so many associations. The clinical importance of these associations becomes especially relevant if lowering plasma total homocysteine by B vitamin treatment can prevent disease and so improve health. Five diseases can at least in part be prevented by lowering total homocysteine: neural tube defects, impaired childhood cognition, macular degeneration, primary stroke, and cognitive impairment in the elderly. We conclude from our review that total homocysteine values in adults of 10 μmol/L or below are probably safe, but that values of 11 μmol/L or above may justify intervention. Homocysteine is more than a disease biomarker: it is a guide for the prevention of disease.

Keywords: B vitamins, cardiovascular disease, cognitive impairment, dementia, stroke.

Abbreviations: AD, Alzheimer's disease; AMD, age-related macular degeneration; CAD, coronary artery disease; CDR, clinical dementia rating; CSPPT, China Stroke Primary Prevention Trial; CVD, cardiovascular disease; GFR, glomerular filtration rate; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; mo, months; MTHFR, methylenetetrahydrofolate reductase; NSAID, nonsteroidal anti-inflammatory drug; NTD, neural tube defect; tHcy, total homocysteine.

Introduction

At the end of 2020, a search of the PubMed database yielded more than 26 000 hits for 'homocysteine'. How did it all start? Fifty years ago, Kilmer McCully first described the vascular pathology of homocystinuria [1]. He noted that thromboembolic disease was a characteristic feature of the inborn errors termed homocystinuria independent of the site of the metabolic defect, pointing to homocysteine as causal risk factor. This is the basis for his homocysteine theory of atherosclerosis, which implies that moderately elevated homocysteine may be a cardiovascular disease (CVD) risk factor also in the general population.

The concept of homocysteine as a risk factor for CVD has, over the past half-century, been dramatically extended by epidemiological studies so that it is now thought to be a risk factor in a large number of diseases or conditions, from conception to death. In general, it is safer to consider raised tHcy as a biomarker since the term 'risk factor' often implies a causal link. A causal link requires much more solid evidence, typically fulfilling the Bradford Hill criteria of causation [2]. Most important, data from clinical trials should demonstrate that lowering homocysteine will prevent a disease. The purpose of this opinion piece is to list the diseases for which homocysteine is considered to be a prognostic biomarker and to describe the evidence that lowering homocysteine can, in some cases, prevent certain diseases. The diseases to be discussed under the prevention theme are neural tube defects (NTDs), impaired childhood cognition, macular degeneration, stroke and cognitive impairment or dementia.

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Homocysteine as a disease biomarker

Raised plasma total homocysteine (tHcy) has been associated, in cross-sectional and prospective studies, with more than 100 diseases, syndromes or outcomes, as shown in Table 1 (details and references in Table S1). In accordance with convention [3], we use the abbreviation tHcy for plasma, or serum total homocysteine, which includes the following forms: free homocysteine (the thiol), homocystine (the disulphide), mixed disulphides and protein-bound homocysteine (bound to cysteine residues in proteins) [4]. (Note that homocysteine bound to nitrogen in lysine residues of proteins (N-homocysteinylation) [5] is not included in this definition.) The range of diseases is remarkable and probably unprecedented for a risk factor or biomarker. The associations do not establish causality, let alone that tHcy is the only cause, but they generate the hypothesis that, for each disease, raised tHcy, reflecting raised intra- and extracellular homocysteine levels, might be one of the component causes in a multifactorial disease [6]. Since most diseases have multiple causes, it is perhaps not surprising that different factors might interact: a good example of this is the synergistic effect of raised tHcy and hypertension on the risk of cardiovascular disease and in particular of stroke [7,8]. An alternative view is that tHcy is merely a biomarker for another cause or causes; for example, it has been suggested that raised tHcy might be a marker for poor lifestyle [9] or impaired renal function [10]. Both views are valid: for some diseases homocysteine may indeed be a 'component cause' [11], but raised tHcy might also be a biomarker for other causes of the same disease, such as impaired methylation, impaired B vitamin status or increased levels of downstream products, such as cysteine. Since the status of B vitamins (folate, vitamins B6, B12 and B2) strongly influences the concentration of tHcy [9,12,13], a key question is as follows: Are diseases associated with raised tHcy caused by homocysteine itself or are they caused by the low levels of one or more B vitamins? Trials in which B vitamins are administered to lower tHcy cannot alone answer this question but they do allow us to test disease preventive strategies, which will be the main topic to be discussed below.

In addition to diseases, raised tHcy is associated with increased all-cause mortality. A marked concentration-related association was reported in an early study by Nygård et al. [14] who found that

Table 1 *Plasma total homocysteine as a disease biomarker*

Disease/outcome
Insufficient B vitamin status
Folate, B12, B6, B2
Inborn errors of homocysteine and vitamin metabolism and transport
Cardiovascular diseases
Myocardial infarction
Severity of coronary artery disease
Restenosis of coronary arteries and adverse outcomes after angioplasty
Vascular calcification
Heart failure
Cardiac hypertrophy
Hypertension
Stroke
Stroke mortality
Silent brain infarct
Carotid plaque area, stenosis, intima–media thickness
Intracerebral arterial stenosis
Peripheral vascular disease
Venous thrombosis
Arterial aneurysm
Arterial stiffness
Atrial fibrillation
Cerebral small vessel disease
Cerebral microbleeds
Disruption of blood–brain barrier
Endothelial mediated dilatation – impaired
Vascular complications of diabetes
Raynaud's syndrome
Takayasu arteritis
Thromboangiitis obliterans (Buerger's disease)
Moyamoya disease
Behçet disease
Erectile dysfunction
Other outcomes
Mortality
Frailty in elderly
Muscle strength, impaired
Sarcopenia
Physical function, gait speed – impaired
Intrinsic capacity (WHO), impaired

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Table 1 (*Continued*)

Disease/outcome
Cancer
Metabolic syndrome
Obesity
Bone disease, osteoporosis
Inflammatory bowel disease, Crohn's disease
Gluten-sensitive enteropathy (celiac disease)
Nonalcoholic fatty liver disease
Renal insufficiency, chronic kidney disease
Chronic obstructive pulmonary disease
Alcohol abuse
Alcohol-withdrawal seizures
Psoriasis
Vitiligo
Sclerosis
Sickle cell disease
Burning mouth syndrome
Atrophic glossitis
Quality of life in centenarians
Obstructive sleep apnoea
Hypothyroidism
Polycystic ovarian syndrome
Telomere shortening
Systemic lupus erythematosus (SLE)
Dermatomyositis
Inflammatory response
Periodontal disease
Hearing loss
Gout
Blood lead concentration
Diabetic neuropathy
Cellular senescence; impairment of autophagy
Maternal tHcy
Pregnancy complications
Outcomes in child
Small for gestational age, foetal growth
Neural tube defects
Congenital heart disease
Orofacial clefts
Renal function
Child cognition, impaired
Child behaviour, impaired
Schizophrenia
Autism spectrum disorder

Table 1 (*Continued*)

Disease/outcome
Central nervous system diseases
Incident Alzheimer's disease/dementia
Vascular dementia, vascular cognitive impairment
Poststroke cognitive impairment
Cognitive decline after concussion
Cognition in infants and children, impaired
Cognition in elderly, impaired
Initiation of cognitive decline in ageing
Conversion from cognitive impairment to dementia
Cognitive decline in dementia
Atrophy of brain tissue/grey matter
Atrophy of brain white matter
White matter damage
Alzheimer brain pathology (P-tau)
Multiple sclerosis
Cognitive decline in Parkinson's disease
Depression
Bipolar disorder
Anxiety
Obsessive–compulsive disorder (OCD)
Post-traumatic stress disorder (PTSD)
Schizophrenia
Amyotrophic lateral sclerosis/ motor neuron disease
Multiple system atrophy
Motor development in infant, impaired
Disruption of blood–brain barrier
Early neurological deterioration after stroke
Glasgow coma scale
Migraine
Autism spectrum disorder
Ocular diseases
Macular degeneration
Ectopia lentis
Retinal vascular occlusion
Retinal arteriosclerosis
Diabetic retinopathy
Exfoliation syndrome and glaucoma
Nutritional blindness

The table lists diseases and syndromes for which there are reports of association with raised total homocysteine. Most of the reports are of prospective studies. Table S1 in the Supplementary files gives references to key original papers and reviews.

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CVD patients with tHcy > 20 μmol/L had a 4.5-fold greater risk of dying than those with tHcy < 9.0. This finding has been confirmed several times since, as reviewed by Fan et al. [15], who found in a meta-analysis that the risk of mortality increased by 33.6% for each 5 μmol/L increase in tHcy levels. A recent report on a cohort of 2,968 CVD patients found that the hazard ratio for death was almost three times higher for those in the top quartile of tHcy (>15.6 μmol/L compared with those in the first quartile of tHcy) (<9.8 μmol/L) [16]. These striking results are illustrated in Figure 1.

Mendelian randomization studies

Rather than measure tHcy in blood samples, several studies have applied the method of Mendelian randomization using polymorphisms known to influence the concentration of tHcy to search for associations with different diseases. This powerful technique indirectly assesses lifetime exposure to tHcy and should be less prone to confounding than direct measurements. However, the results need to be interpreted with care.

The commonest example in the homocysteine field is the use of the *MTHFR* 677 C → T polymorphism. The presence of two T alleles is usually associated with lower folate levels and an elevated concentration of tHcy, but the effect on tHcy is influenced by several covariates such as age, sex, race and folate intake [17]. In most studies, the effect of TT on tHcy is more marked at low population folate status [9,18,19]. The influence of these covariates is often ignored in Mendelian randomization studies. Another factor that was not always recognized is that several early studies on MTHFR were underpowered to detect a significant effect [20]. A well-powered meta-analysis by Wald [21] on about 12,000 cases and 12,000 controls found that people with the TT genotype had a greater risk of CVD. In Wald's analysis, 33 out of the 46 studies also reported tHcy levels, which allowed the authors to estimate that TT carriers, for a 5 μmol/L increase in tHcy, had an odds ratio of 1.42 (1.11, 1.84) for ischaemic heart disease. Subsequent reports did not always confirm the association; for example, Clarke et al [22] did a meta-analysis of 19 unpublished data sets that included 48,175 coronary artery disease (CAD) cases and 67,961 controls with information on the *MTHFR* 677 C → T polymorphism and found a nonsignificant odds ratio for CAD comparing TT subjects with CC subjects of 1.02 (0.98, 1.27). This led the authors to conclude that lifelong moderate tHcy elevation has little, or no, effect on CAD.

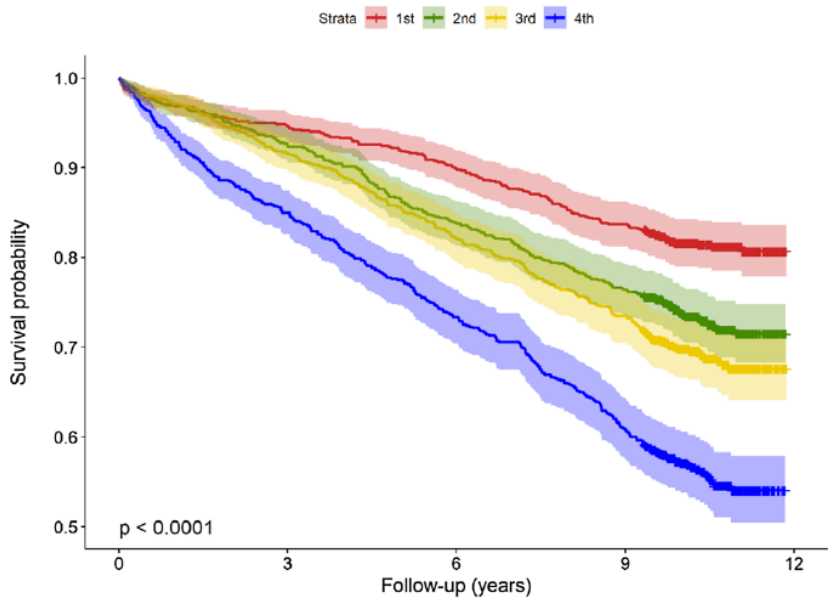

Fig. 1 *Kaplan–Meier plots showing survival probability according to quartiles of tHcy in a cohort of 2,968 CVD patients. Quartiles shown are as follows: 1st, red; 2nd, green; 3rd, yellow; and 4th, blue. The 4th quartile was tHcy > 15.6 μmol/L. From Pusceddu et al. (fig. 1)[16].*

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However, as stated by the authors, they made a crucial assumption: '*These datasets did not include measurements of blood homocysteine, but homocysteine levels would be expected to be about 20% higher with TT than with CC genotype in the populations studied*'. To use phenotypic data, that is tHcy measurements, from different data sets to those used for the genetic studies, is not sound science [23], in particular given how much tHcy has changed over the last few years due to widespread use of mandatory and voluntary folic acid fortification. Thus, we question the validity of the main conclusion about tHcy and CAD based on their finding in the Mendelian randomization study [22].

Interestingly, another Mendelian randomization study on *MTHFR* was reported from the United States that covered the period before and after the introduction of mandatory folic acid fortification [24]. Overall, the TT genotype was associated with a significantly *lower* CVD mortality. However, this effect of the TT genotype only occurred in the period *after* the introduction of mandatory fortification, that is when the genotype effect on tHcy is limited. In contrast, before fortification, the pattern was in the expected direction, with (nonsignificant) higher risk in the TT genotype. The authors speculated that this paradoxical result may be that other CVD risk factors have a stronger effect in the CC and CT genotypes [18] in a population with high folate status.

Three other large-scale Mendelian randomization studies deserve attention: first, a report on 31,400 subjects with CAD and 92,927 controls of European descent in which 18 polymorphisms were identified that influenced the concentration of tHcy [25]. In this study, no association was found between the 18 polymorphisms at 13 loci that influenced tHcy and the risk of CAD. This finding led the authors to conclude that the results 'provide further refutation of the causal relevance of moderately elevated tHcy concentrations to CAD in white populations'. However, all these 18 genetic polymorphisms only accounted for 5.9% of the variation in tHcy levels. Furthermore, as pointed out by Lehmann and Cortina-Borja [26], 8 out of the 18 polymorphisms were associated with lower levels of tHcy yielding a total additive effect of 0.109 standard deviations of tHcy in a standard normal scale, which is a small shift in the normalized distribution that is not going to be clinically significant. We therefore do not think that the conclusion of van Meurs et al. [25] can be justified.

Two more Mendelian randomization studies examined the relation of the same polymorphisms as above [25] with Alzheimer's disease (AD) in different populations and concluded that there is no causal relation between tHcy and AD [27,28]. These sets of authors assumed, without evidence, that the effect of the genetic polymorphisms on tHcy would be the same in the different patient populations. The same criticisms as above can be applied to these reports, and it can be concluded that the use of Mendelian randomization in these two studies was based on genetic scores with limited predictive ability and so was probably inappropriate [26].

Overall, we do not think that Mendelian randomization has much to contribute to the homocysteine field since the mutations studied have such a limited effect on the concentration of tHcy. Further concerns have been expressed about the use, or misuse, of the Mendelian randomization method in general [29,30] and in relation to tHcy in particular [31].

What causes raised plasma homocysteine?

This topic will not be reviewed here, but we will mention some of the main reasons why tHcy becomes elevated. There are many genetic, physiological, lifestyle and clinical conditions that determine tHcy, which are listed in Table 3 of our earlier review [4]. We can now add air pollution [32], whose effect is enhanced at low B vitamin status [33]. Probably, the most common significant cause of raised tHcy is insufficiency or impaired function of one or more of the B vitamins (folate, B12, B6 and B2) involved as coenzymes in its metabolism – for references, see Table S1. But another important cause of raised tHcy is renal impairment since tHcy is strongly and linearly inversely related to glomerular filtration rate (GFR); as GFR falls below about 60 mL/min, the level of tHcy begins to increase [10,34,35]. Diabetes associated with nephropathy is also associated with hyperhomocysteinaemia, due to impaired methylation and transsulphuration [36]. Of the metabolic causes, one of the most important is methylation demand: quantitatively, two of the most important methyltransferases in this regard are guanidinoacetate methyltransferase (that produces creatinine) and phosphatidylethanolamine N-methyltransferase

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(that produces phosphatidylcholine) [37]. In addition, a large number of drugs can influence tHcy levels; a list is given in Table 4 of our review [4], to which we can add isotretinoin [38]. A comprehensive survey [39] screened over 82 biochemical and haematological markers in the US population to identify those that were associated with high tHcy (>14 μmol/L). The following nine markers were positively associated with raised tHcy: low serum folate, low serum B12, age, creatinine, serum uric acid, alkaline phosphatase, cotinine, mean cell haemoglobin and red cell distribution width.

This review deals with modestly raised levels of tHcy and does not cover the very high levels that can occur with inborn errors of amino acid and vitamin metabolism or transport, for which some key references are given in Table S1.

How does raised homocysteine cause disease?

When we consider a possible role of raised tHcy in the causation of disease, we have to be aware that several of the causes of raised tHcy, notably age, renal impairment, air pollution and B vitamin insufficiency, are themselves associated with an increased risk of disease. In that sense, tHcy might be one component of the sufficient cause(s) of the disease [11] for the outcomes listed in Table 1, or alternatively the disease might occur independently of raised tHcy.

A large variety of possible mechanisms has been proposed to explain the link between raised tHcy and one or more diseases. These will not be described here but are discussed in several reviews [5,40–49]. It is likely that more than one mechanism can be at work at the same time, which may explain why raised tHcy is such a powerful risk factor for some diseases.

We briefly mentioned N-homocysteinylation above. N-homocysteinylation alters the properties of the protein and has been associated with several disease outcomes, as reviewed by Jakubowski [5]. This process is irreversible and cumulative. Thus, homocysteinylation of long-lived proteins may explain some of the progressive clinical manifestations in age-related conditions [50]. An example is the N-homocysteinylation of the protein tau, which occurs in Alzheimer's disease (AD) [51] and leads to the dissociation of tau from beta-tubulin. Such a phenomenon could partly account for the gradual development of AD over several decades [52]. Although a potential mechanism for many chronic diseases, this pathway is unlikely to respond to B vitamin treatment if not initiated at an early stage of the disease process.

Homocysteine and disease prevention – clinical trials of B vitamin supplementation

The most robust way of testing whether lowering tHcy can prevent a disease is to carry out a randomized controlled trial of B vitamins. There have been many such trials, some of which are listed in Table 2. Altogether, we have found trials that relate to 17 different diseases or outcomes, from which we have selected five for detailed discussion: those concerning neural tube defects (NTDs), impaired childhood cognition, macular degeneration, primary stroke and cognitive impairment in the elderly.

It might be helpful if we state here the criteria that we think should be adopted when performing and interpreting the results of such trials. The

Table 2 *Lowering homocysteine and disease prevention*

Disease
Cardiovascular disease
General
Restenosis after angioplasty
Stroke: secondary trials
Stroke: primary trial
Carotid–intima thickness
Impaired flow-mediated dilatation
Carotid plaque area
In kidney disease
Hypertension
Macular degeneration
Hearing loss
Celiac disease
Chronic kidney disease
Migraine
Maternal trial: neural tube defects
Maternal trial: child cognition
Maternal trial: congenital heart disease
Cognitive impairment

The table lists diseases or outcomes for which there are reports of intervention trials to lower tHcy.
For references and comments, see Table S2.

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Table 3 *Essential trial criteria that must be fulfilled for concluding whether treatment with B vitamins or lowering raised tHcy can influence a disease outcome*

Risk factor: baseline tHcy or B vitamin status[a]	The risk factor to be treated (elevated tHcy or suboptimal B vitamin status) should be present at baseline so that treatment benefit may occur.
Outcome measurement	Sensitive and appropriate tests must be used for measuring the outcome variable, preferably physical measures that are not influenced by daily variations or subjective evaluations.
Absence, or limited presence, of the outcome variable at baseline	At start of trial, participants should be at risk of the outcome. But participants should not suffer the end stages of a disease where improvement is unlikely and further progression may be limited and difficult to assess.
Trial duration	Should be sufficient to measure a clinically relevant and significant number of events or decline in function in the placebo group.
Vitamin dose and combinations	Simple dietary modification is usually inadequate; a combination of pharmacological doses of B vitamins, especially of B12, is needed, in order to sufficiently lower tHcy.
Sensitivity analysis	The protocol should prespecify analysis according to baseline concentrations of tHcy and/or of B vitamin status.
Efficacy analysis	The protocol should prespecify analysis according to treatment effect on tHcy lowering. Subjects that have conditions that render the B vitamin ineffective (antivitamin drugs, renal failure, malabsorption states, etc.) should be identified.
Subgroup analyses	The protocol should prespecify data analysis according to factors that may interact with the effect of B vitamin treatment, for example CVD risk factors, omega-3 fatty acids, other known genetic and nongenetic risk factors, and antiplatelet drug use.

[a]In this table, the term 'B vitamins' means those that are directly required for homocysteine metabolism, that is folate, vitamin B12 and vitamin B6. Vitamin B2 may also influence homocysteine indirectly via its role as cofactor for MTHFR.

consensus report on homocysteine and dementia presented a list of ideal criteria for trials in dementia [53], and these have been generalized in Table 3. The extent by which such criteria have been adopted in published trials will be considered in our comments below. Critically, the interpretation of the results must take into account that the effect of B vitamins may be very different in different populations or subgroups of the population, as most convincingly demonstrated in the China Stroke Primary Prevention Trial (CSPPT, to be described below) [54].

Homocysteine and disease prevention – neural tube defects

A poor folate and/or B12 status is associated with an increased risk of NTDs [55], which is therefore likely to be caused by dysregulation of 1-carbon metabolism. In the year that the successful MRC trial of folic acid to prevent recurrent neural tube defects was published [56], a report appeared suggesting that homocysteine metabolism might be abnormal in women who had had baby with an NTD [57]. Subsequent studies, with blood samples taken during or after pregnancy, have on the whole reported modestly raised tHcy levels in the mother (Table S1) [58]. Preferably, we need further studies on blood samples taken before conception.

Based on existing data, we can see, indirectly, the possible effect of lowering tHcy on the incidence of NTDs, that is by comparing the change in NTD prevalence with the change in tHcy levels after the introduction of mandatory folic acid fortification. In the United States, the Center for Disease Control found a decrease of 27% in the average annual prevalence of NTDs in the United States from 1995–1996 to 1999–2000 (4,130 cases before

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Table 4 *Interactions between risk factors and folic acid treatment for stroke*

Group/subgroup	Reduction in risk (%)	Interaction P value	References
Hypertension (total population)	21		[110]
Hypertension plus high cholesterol	33	0.024	[112]
Hypertension plus diabetes or glucose > 7	34	0.01	[113]
Hypertension plus high tHcy and low platelets	73	0.004	[114]
Hypertension plus folate-deficient	64	NR	[115]

Interactions between risk factors and the protective effect of folic acid treatment in addition to enalapril on the occurrence of first stroke in the CSPPT. NR, not reported.

fortification and 3,020 after fortification) [59]. The change in tHcy levels in the United States over this period is shown in Figure 2.

After fortification, the median concentrations of tHcy did not decline very much in the whole population, and in women of child-bearing age, they only fell by 3.6 %, from 7.02 to 6.77 μmol/L, but the proportion of women with tHcy above 13 μmol/L fell by 72% from 9.68% to 2.69% [60]. It is possible, therefore, that any benefit of lowering tHcy concentrations is due to the decrease in the proportion with high levels.

The question we cannot answer is as follows: Was the 27% decline in NTDs related to the 72% decline in concentrations of tHcy > 13 μmol/L? One way of approaching this question is to see whether the very different changes in NTDs in various Canadian provinces after fortification in 1998 (Figure 3) could be related to the prefortification tHcy levels. We have been unable to obtain the tHcy values for the different provinces, but there are two studies for Newfoundland. One reported that the median tHcy in women at their first antenatal visit declined by 19.6 % after fortification, from a value of 8.53 to one of 6.86 μmol/L. The data show a particularly large decline in tHcy in women with high baseline tHcy concentrations [61]. The second study was on women of child-bearing age and reports a 9.8% decline in the mean tHcy but a 52.2% decline in the proportion of women with tHcy > 13.2 μmol/L [62].

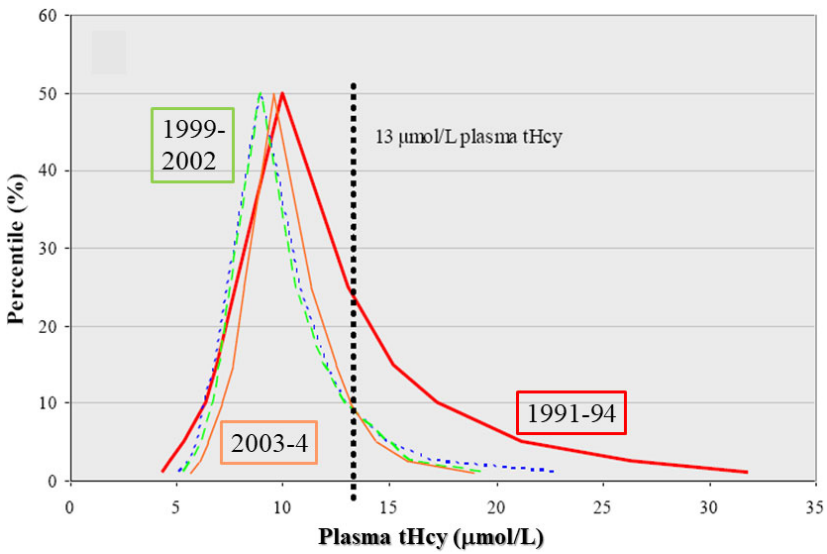

Fig. 2 *Distribution of plasma tHcy levels in the entire population in the NHANES study, USA, before fortification (1991-1994) and two periods after fortification became mandatory in 1998. Median values: 1991-1994: 8.11 μmol/L; 1999-2002: 7.51 μmol/L; and 2003-2004: 7.88 μmol/L. Redrawn from fig. 2A in Pfeiffer [60].*

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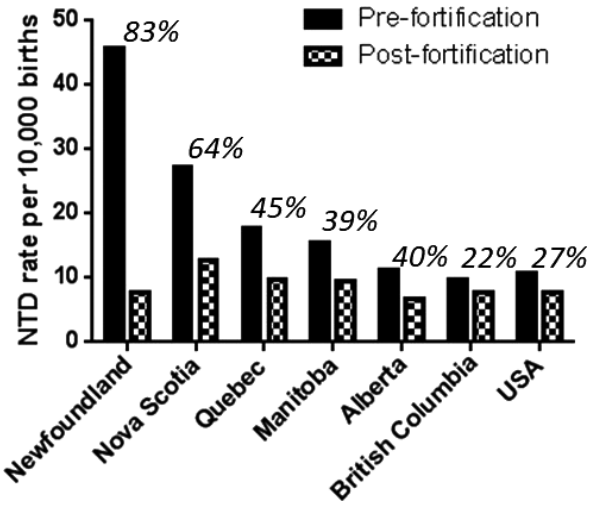

Fig. 3 *Rates of neural tube defects in Canada and the United States before and after introduction of mandatory folic acid fortification in 1998. The figure is plotted from the data published by De Wals et al. for Canada [63] and by the Center for Disease Control for the United States [59].*

Over the same period, the prevalence of NTDs fell by 78% in Newfoundland [61,62], close to that shown in Figure 3. It would be of great interest to have similar data for British Columbia.

There is thus a *prime facie* case that the fall in NTD prevalence after folic acid fortification might be related to the decline in tHcy levels in women of child-bearing age, but that does not of course exclude a more direct effect of folic acid or of folate-dependent reactions not related to homocysteine.

Evidence consistent with a link to homocysteine comes from China, where there is a marked gradient in the population tHcy with high levels in the north and low levels in the south. The prevalence of hyperhomocysteinaemia (>15 μmol/L) was 34.8% in the north and 16% in the south [64]. There is also a marked gradient in the prevalence of NTDs: in northern China, the prevalence is more than 4 times higher than in the south [65,66]. Supplementation of women with 0.4 mg folic acid daily during the periconceptional period reduced the incidence of NTDs by 79% in the north and by 41% in the south [65]. No measurements of tHcy were reported, and so, this result only provides indirect evidence that lowering tHcy will prevent NTDs.

Another birth defect, congenital heart disease, may be related to maternal Hcy (Table 1), and it is therefore noteworthy that there was a 6.2% decline per year in the incidence of such defects in babies born in Quebec after folic acid fortification was introduced [67].

Homocysteine and disease prevention – impaired infant cognitive development

There are several reports of impairment in the cognitive development of infants whose mothers have elevated tHcy during pregnancy (Table S1), but very few trials have been done to see whether lowering tHcy in the mother can prevent the cognitive impairment in the child. A randomized trial of daily B12 (50 μg) in pregnant women with poor B12 status showed that markers of B12 status in the child are improved, with tHcy falling by half compared with the placebo group [68]. In this trial, infants (30 months) of mothers who had elevated tHcy in the second and third trimesters had lower scores in expressive language, whereas the infants of mothers treated with B12 had higher scores than those treated with placebo [69]. There are retrospective studies on maternal folate intake in the periconceptional period or later and the risk of autistic spectrum disorder in the child, but the results have been inconsistent, and a meta-analysis concluded that there was no beneficial effect [70]. There has, however, been a large cohort study, which found a strong protective effect of maternal folic acid intake against autistic traits in the children of mothers taking anti-epileptic drugs during pregnancy [71]. An observational study on maternal folate intake or levels in relation to child development has reported beneficial effects of good maternal folate status on the child's performance in cognitive tests at age 9-10 years [72], but in another study children (aged 4–5 years) of women who took 1 mg or more of folic acid per day during the periconceptional period suffered impaired cognition in several domains [73]. A recent randomized trial showed that maternal folic acid consumption at a dose of 400 μg/day, during the second and third trimesters, is associated with better cognition at age 3 years and with better word reasoning at age 7 years [74]. None of these studies reported tHcy levels in the mothers, but it is likely that folic acid consumption will have lowered the level, although it is, of course, possible that the effects of folate are independent of tHcy. Clearly, the dose of folic acid is very important and more trials of tHcy lowering in the mother need to be done to find the optimal way of preventing cognitive impairment in their children. The public health significance is

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obvious in an age when folic acid fortification is widely implemented.

Homocysteine and disease prevention – macular degeneration

Age-related macular degeneration (AMD) is a major cause of blindness and exists in nearly 200 million people worldwide, a number predicted to increase to almost 300 million by 2040 [75]. Elevated tHcy has been associated with increased risk [76], but more recent studies have, however, cast some doubt on the association [77,78]. Nevertheless, there is evidence from prospective studies that the supplementary intake of B12 [79] and total folate intake [80] may be protective against the development of AMD. Importantly, a randomized trial on 5,205 women with CVD or CVD risk factors found that supplementation with folic acid (2.5mg), B12 (1 mg) and B6 (50 mg) led to a 34% reduced risk of any AMD and a 41% reduced risk of visually significant AMD over a 7.3-year period [81]. This trial has potential important public health implications. Even though no attempt at replication has been reported, this result should lead ophthalmologists to consider recommending B vitamins to those with, or at high risk of, AMD.

Homocysteine and disease prevention – stroke

Historical background

Raised tHcy is a likely risk factor for the different forms of stoke: large and small vessel disease and even haemorrhagic stroke (Table 1 and Table S1). Many randomized trials have tested whether lowering tHcy by supplementary B vitamins will reduce the risk of stroke. The majority of these trials have been *secondary*, that is carried out in patients who have had a stroke or a CVD event. Most trial results have been disappointing. The prevailing view after publication of the VISP [82], HOPE [83] and NORVIT [84] trials was that lowering tHcy was not able to prevent a second stroke and other CVD events. The principle investigator of NORVIT, K. H. Bonaa, said at a press conference at the European Society for Cardiology in Stockholm in 2005: 'The homocysteine hypothesis is dead. Homocysteine in not a causal risk factor. It is an innocent bystander'.

The idea that lowering tHcy does not prevent further CVD events in patients who have already suffered a cardiac event or stroke became widely accepted following a series of reviews and meta-analyses (see, for example, Ref. [85–88]). It almost became a dogma. One author asked whether the idea that lowering tHcy might prevent CVD was 'just another distraction' [89]. This attitude may have been the reason why positive evidence of a beneficial effect was generally ignored. An example is the original report of the HOPE-2 trial [83]. The authors of this report concluded: 'Supplements combining folic acid and vitamins B6 and B12 did not reduce the risk of major cardiovascular events in patients with vascular disease'. This conclusion was made in the abstract in spite of the statement just above it: 'Fewer patients assigned to active treatment than to placebo had a stroke (relative risk, 0.75; 95 per cent confidence interval, 0.59 to 0.97)'. This finding of a 25% reduced risk for stroke was downplayed in the report, and the Kaplan–Meier plot showing the result was relegated to an online supplement. We were so surprised that we submitted a Letter to the journal highlighting this important result for stroke, and we reproduced the Kaplan–Meier plot, which was published in print by the journal [90]. The HOPE-2 report provides a good example of the thinking at the time. In their original report of 2006, the authors wrote:

> From a biologic perspective, a treatment benefit restricted to stroke would be difficult to explain.... Therefore, we believe that the apparent beneficial effect of B vitamin supplements on stroke in our trial may represent either an overestimate of the real effect or a spurious result due to the play of chance

The view that stroke and myocardial infarction are biologically similar is surprising since there are several fundamental differences, which Spence has discussed [91].

In the period after Bonaa's statement in 2005 and the report of the HOPE-2 trial in 2006, views about tHcy lowering and stroke began to change, mainly because of recognition of the importance of studying the effect of B vitamin treatment in different subgroups – see the historical survey by Spence [92]. Three landmark studies can be identified that led to this change: the first study was by Spence et al. in 2005 [93]; the second was by Saposnik et al. in 2009 [94]; and the third was by Wald et al. in 2011 [95].

Spence et al. [93] re-analysed the VISP trial after excluding patients who were likely to have B12 malabsorption and patients with renal impairment

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(<10th percentile of GFR), with the view of excluding patients that were unlikely to respond to the intervention (see penultimate criterion in Table 3). In this subgroup, baseline B12 status and the dose of B12 administered had important effects such that the best event-free survival was obtained in patients with good B12 status (> median) who received a high dose of B12 (400 μg). Thus, B12 status was a key determinant of the response to vitamin therapy in this trial, conducted in a country with mandatory folic acid fortification. The crucial importance of B12 was emphasized in an editorial by Spence [96] that was entitled 'Homocysteine - call off the funeral'. Here, Spence stated 'in the elderly (who represent a large proportion of vascular patients), the key nutritional determinant of tHcy [in a country with folic acid fortification] is vitamin B12, and the real problem is malabsorption of B12'. Furthermore, it is inappropriate to define adequate levels of serum B12 by the normal range, because when defined metabolically (by levels of B12 below which methylmalonic acid is elevated) about 20% of the elderly have B12 insufficiency. We have discussed this matter in an editorial [97] and in more detail in a book chapter [98]; in the latter, we estimate that based on a large number of studies, about 30% of elderly suffer from B12 insufficiency.

Although the authors of the original HOPE-2 report of 2006 described the effects of B vitamins in several subgroups, they limited this analysis to the primary outcome and did not look at stroke separately. However, this was done a few years later by Saposnik et al. [94] who found some striking trends. The main findings are shown in Figure 4.

As shown in Figure 4, there was an apparent reduction in the risk of stroke in some subgroups following B vitamin treatment: those who were

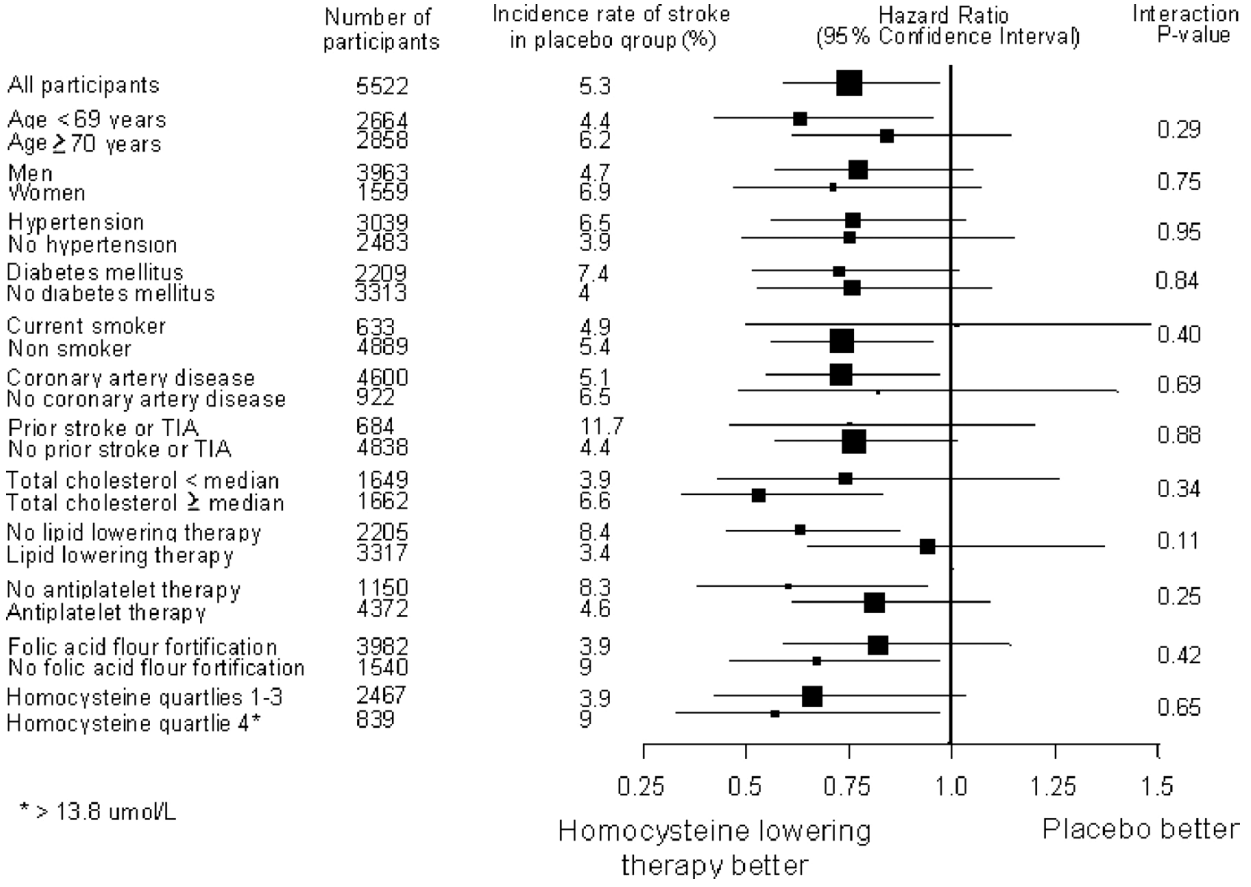
Number of participants
Incidence rate of stroke in placebo group (%)
Hazard Ratio (95% Confidence Interval)
Interaction P-value
All participants 5522 5.3
Age <69 years 2664 4.4
Age ≥70 years 2858 6.2 0.29
Men 3963 4.7
Women 1559 6.9 0.75
Hypertension 3039 6.5
No hypertension 2483 3.9 0.95
Diabetes mellitus 2209 7.4
No diabetes mellitus 3313 4 0.84
Current smoker 633 4.9
Non smoker 4889 5.4 0.40
Coronary artery disease 4600 5.1
No coronary artery disease 922 6.5 0.69
Prior stroke or TIA 684 11.7
No prior stroke or TIA 4838 4.4 0.88
Total cholesterol < median 1649 3.9
Total cholesterol ≥ median 1662 6.6 0.34
No lipid lowering therapy 2205 8.4
Lipid lowering therapy 3317 3.4 0.11
No antiplatelet therapy 1150 8.3
Antiplatelet therapy 4372 4.6 0.25
Folic acid flour fortification 3982 3.9
No folic acid flour fortification 1540 9 0.42
Homocysteine quartlies 1-3 2467 3.9
Homocysteine quartlie 4* 839 9 0.65
* > 13.8 umol/L
0.25
0.5
0.75
1.0
1.25
1.5
Homocysteine lowering therapy better
Placebo better

Fig. 4 *Risk of any stroke according to baseline participant characteristics, comparing homocysteine-lowering therapy vs. placebo. The size of each symbol is proportional to the number of patients in each subgroup. From [94], with permission.*

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younger than 69 y, those with higher baseline tHcy or cholesterol levels, those from countries without folic acid fortification and those not taking antiplatelet or lipid-lowering drugs. Although there were no statistically significant differences in stroke risk between the subgroups, the trends are apparent. Noteworthy is that the reduction in stroke risk was the greatest in the subgroups with high baseline tHcy and with high cholesterol: the HR for those in the top tertile (>13.8 μmol/L) of tHcy was 0.57 [0.33, 0.97]. These results may reflect the fact that as a risk factor for CVD, raised tHcy is known to interact with other established risk factors [7].

The third landmark study, by Wald et al. [95], recognized that, in secondary trials, nearly all participants will be taking several types of medication in line with current medical practice, and so, the question has to be asked: Will lowering tHcy add further protection to, or interact with, existing treatments? Wald et al. [95] asked the question whether the use of antiplatelet drugs might influence the effect of lowering tHcy on the risk of CAD events. They found that in trials with the *lowest* proportion of patients (mean 60%) taking antiplatelet drugs, the effect of tHcy lowering tended to be protective with a relative risk of 0.94 (0.84, 1.05), whereas in trials with a high proportion (mean 91%) taking antiplatelet drugs, the relative risk was 1.09 (1.00, 1.19). This report, together with that of Saposnik et al. [94], led to re-examination of several trials, with notable consequences. For example, the large VITATOPS trial originally reported no beneficial effect of B vitamins on secondary stroke [99], but a re-analysis of the data found that patients who were not taking antiplatelet drugs had a 24% reduction in the risk of a primary event (major CVD event or CVD death) [100]. A meta-analysis of three trials (VISP, HOPE-2, VITATOPS), on 4,643 vascular patients not taking antiplatelet drugs, used stroke as the primary outcome and found an overall 29% reduction in stroke risk in those taking B vitamins [101]. These results have important practical implications since they support the use of tHcy-lowering B vitamins in people with vascular disease who are not taking antiplatelet drugs. Indeed, there was evidence from the VISP trial that those taking antiplatelet drugs might be at greater risk of stroke (HR = 1.43, 1.02, 2.01) when treated with high-dose B vitamins [102] and so high-dose B vitamins are probably contra-indicated in such patients. An important question is whether the unique effect of antiplatelet drugs on B vitamin treatment is confined to aspirin. If so, this may have strong implications for treatment, in particular given the evidence that aspirin has limited effect on CVD events in older subjects [103].

Importance of subgroups

Whether the findings of these three landmark studies justify a change in medical practice is still controversial, but they provide strong hypotheses that can be tested in future studies. These hypotheses are based upon the recognition that subgroups are important in medicine and in research. Rather than automatically reject subgroup analysis of clinical trials as some scientists do, Spence and Stampfer [104] argued: 'Subgroup analyses founded in biology may have the potential to importantly inform interpretation of clinical trial results, provided sufficient caution is exercised. Thoughtfully derived subgroups, especially those formed *a priori* and not derived *post hoc* from the same data set, can stimulate further work and reinterpretation of existing data'.

Let us look at some analyses of stroke outcomes of tHcy-lowering trials that are based on the definition of one or more subgroups. Wang et al. in a meta-analysis in 2007 [105] found an overall risk reduction for stroke of 18% but that benefit was confined to subjects in nonfortified countries (risk reduction of 25%). There was also a risk reduction by 23% in those whose tHcy level fell by > 20%. Perhaps unsurprisingly, these authors found a greater risk reduction in those treated with B vitamins for more than 36 mo (29% risk reduction). In 2012, a meta-analysis by Huo et al. [106] confirmed the importance of folic acid fortification, finding a RR of stroke of 1.03 [0.88, 1.21] after B vitamin treatment in countries with fortification, but a significant ($p = 0.01$) RR of 0.89 [0.82, 0.97] in countries without fortification. Importantly, Huo et al. [106] also confirmed Saposnik's finding [94] that the use of lipid-lowering drugs modified the response to B vitamin treatment: in trials with a high proportion of statin users, there was no benefit, but in trials where < 80% used statins, there was a 23% reduction in risk ($p = 0.005$). Saposnik [94] also found a greater treatment benefit in patients aged < 69 years, from regions without folic acid fortification, with higher baseline cholesterol or homocysteine levels, and in those not receiving antiplatelet drugs at enrolment. The effect of living in countries with mandatory folic

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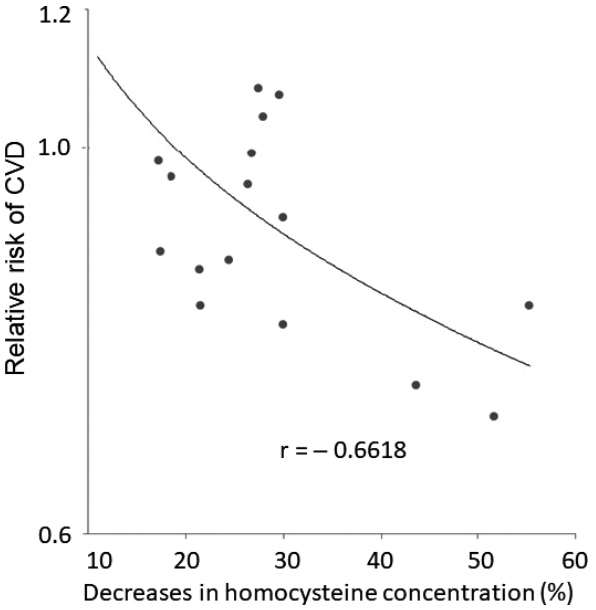

Fig. 5 *Relative risk of CVD in relation to percentage decreases in tHcy concentration based on 16 trials with full records of tHcy changes after the intervention. Note the r value of −0.6618. From Li (fig. 6) [108].*

acid fortification was confirmed in a meta-analysis in 2015 on 14 trials with 39,420 patients [107]: these authors found a relative risk of 0.88 [0.77, 1.00] in those without fortification compared to 0.94 [0.58, 1.54] in those with fortification. The difference was just significant ($p = 0.05$). The authors also reported the fall in tHcy levels in the two groups after B vitamin treatment: in the nonfortified group, tHcy declined by 27%, whereas in the fortified group, it only declined by 18.4%. An important meta-analysis in 2016 by Li et al. [108] on 30 trials of B vitamin treatment in > 80,000 patients found an overall 10% reduced risk of stroke. But the risk reduction was 21% in those with lower baseline serum folate (<16 nmol/L); 15% in those living in countries without folic acid fortification; and 19% in those ≤ 60 years. In this meta-analysis, there was no beneficial effect on the risk of CAD, but there was a small, but significant effect on total CVD with an overall relative risk of 0.96 [0.92, 0.99] in the B vitamin group. Notably, there was a significant inverse dose–response relationship between the fall in tHcy after treatment and the risk of CVD (Figure 5). This result is consistent with a causal role of homocysteine in CVD.

The authors of this comprehensive Harvard meta-analysis concluded as follows: 'Our findings provide support for a modest benefit of folic acid supplementation for the prevention of stroke. There was a 10% reduced risk of stroke and a 4% reduced risk of overall CVD with folic acid supplementation. A greater benefit for CVD was observed among participants without pre-existing CVD or with lower plasma folate levels at baseline and in studies with a larger decrease in homocysteine levels. We did not observe any significant benefit or harm with folic acid supplementation for the risk of CAD' [108].

In the light of the importance of baseline tHcy or B vitamin status for outcome, it is noteworthy that another meta-analysis showed a greater beneficial effect folic acid on the progression of atherosclerosis, as measured by the carotid–intima thickness, in trials with greater fall in tHcy levels [109].

Primary prevention of stroke: the China Stroke Primary Prevention Trial (CSPPT)

All the trials discussed above have been secondary trials in patients with CVD. The only large primary trial of homocysteine-lowering treatment has been conducted in China [110]. The results of this important trial will be summarized, with particular focus on the effect of treatment in different subgroups. The trial included 20,702 patients with hypertension (mean systolic BP 167 mm Hg), but without a history of stroke or myocardial infarction, with a median tHcy of 12.5 μmol/L, median serum folate of 18.35 nmol/L and median B12 of 280 pmol/L. They were randomized equally into a group treated with the antihypertensive drug enalapril (10 mg) and a group treated with enalapril and 0.8 mg folic acid. After a median treatment period of 48 mo, the trial was terminated early due to a significant efficacy difference in the primary outcome of first stroke, with 355 (3.4%) strokes in the enalapril-only group and 282 (2.7%) in the enalapril and folic acid group, giving a hazard ratio of 0.79 (95% CI 0.68,0.93), $p = 0.003$. Figure 6 shows the Kaplan–Meier curves. In relation to our discussion above, it is important that only a small proportion (3.1%) of the participants in the CSPPT were taking antiplatelet drugs and only 0.8% were taking lipid-lowering drugs.

Further analyses showed that the beneficial effect was limited to the prevention of ischaemic stroke (HR: 0.76 [0.64, 0.91]), and not haemorrhagic

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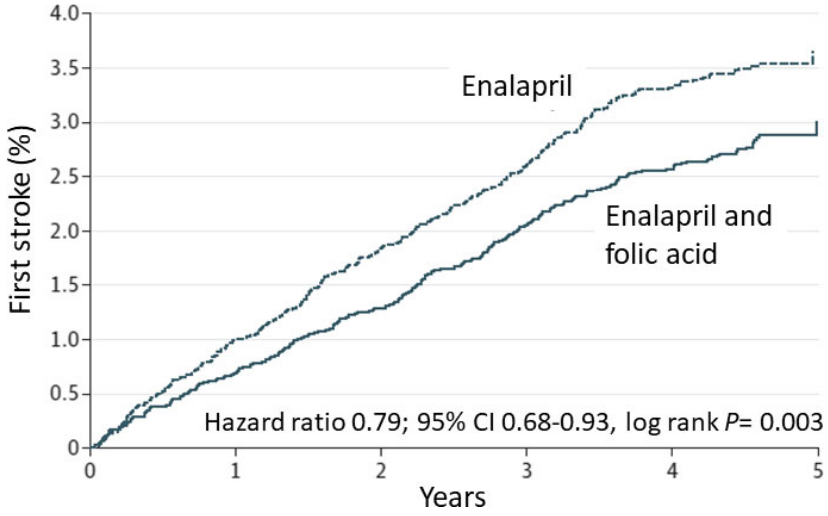

Fig. 6 *Cumulative hazards of first stroke by treatment group in the CSPPT. From Huo et al. 2015 (fig. 2) [110].*

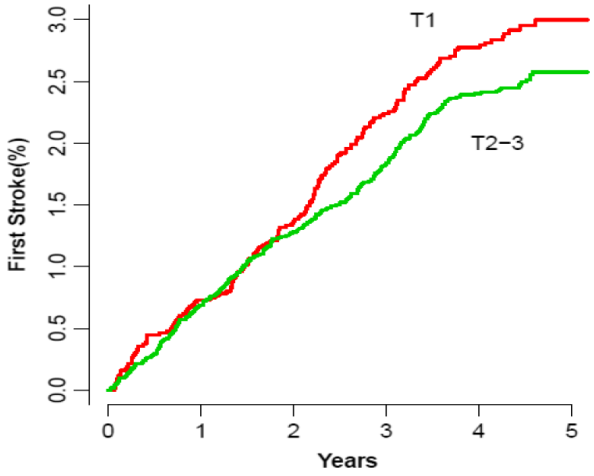

Fig. 7 *Kaplan–Meier survival curves of the cumulative event rate of first stroke by tertiles of per cent decline in tHcy in the CSPPT (with permission, from fig. e-2, ref. [111]).*

stroke or myocardial infarction. Subgroup analysis showed a greater protection against stroke in those with low baseline folate levels of < 12.7 nmol/L (HR: 0.61 [0.45, 0.82]) compared with higher folate and in those with higher baseline tHcy levels of 12.5–15.5 μmol/L (HR: 0.71 [0.53, 0.96]) compared with lower tHcy.

A *post hoc* analysis of the CSPPT data has shown that the degree of lowering of tHcy is a useful marker that predicts the effectiveness of folic acid in preventing first stroke [111]. Participants who had a first stroke had a significantly smaller fall in tHcy than those who did not have a stroke. Those in tertiles 2 and 3 of the decline in tHcy had a lower stroke risk than those in tertile 1 (Figure 7) with a HR of 0.79 [0.64, 0.97. Per 20% fall in tHcy, there was 7% reduction in the risk of stroke (HR: 0.93 [0.90, 0.97]).]. The association between the decline in tHcy and reduction in stroke risk was independent of baseline tHcy and folate levels and blood pressure control. These results add further support for the view that homocysteine itself is one of the causal factors for stroke.

Subgroups in the CSPPT

The CSPPT has extensively examined the importance of subgroups in the clinical response to lowering tHcy, as we will now summarize.

Analysis of the relationship between baseline tHcy and the outcome of the CSPPT led to a remarkable finding: the baseline platelet level markedly influenced the response to folic acid: only those in the lowest quartile of platelets benefitted. In patients in the first platelet quartile with tHcy < 15 μmol/L, there was a 51% reduction in stroke risk, and in those with baseline tHcy ≥ 15 μmol/L, there was a 73% reduction in stroke risk (HR: 0.27 [0.11, 0.64]). In contrast, there was no significant reduction in risk in those treated with folic acid with either low or high tHcy and platelets in the second and higher quartiles [112]. The authors speculated that the combination of low platelets and high tHcy

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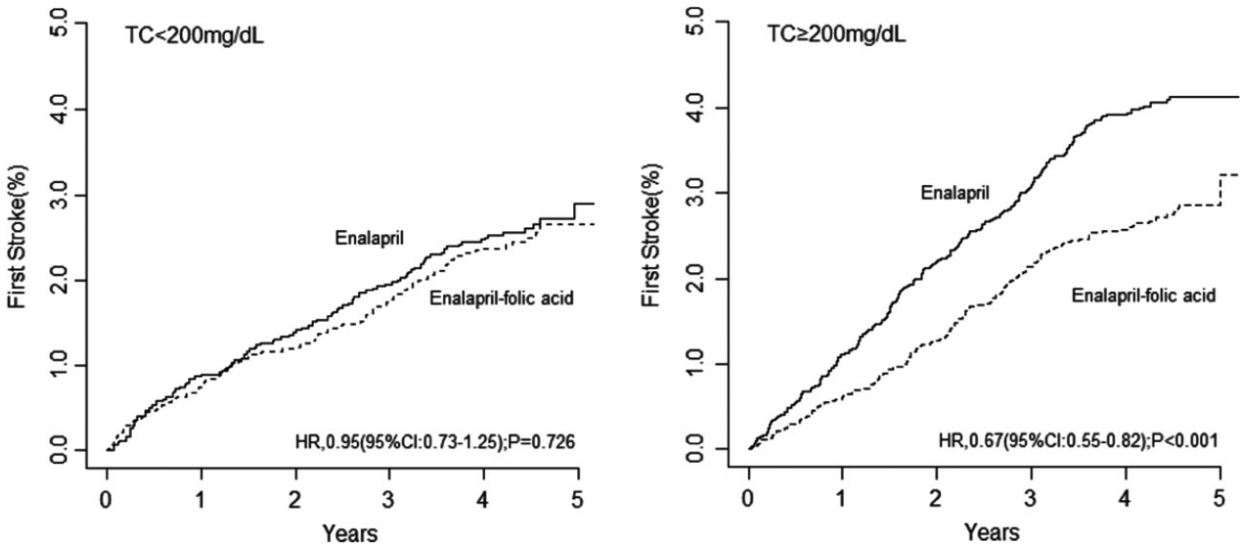

Fig. 8 *Kaplan–Meier curves of cumulative hazards for first stroke within each baseline total cholesterol (TC) strata (from fig. 2, ref [112]).*

might be a marker for endothelial injury, platelet adherence and consumption and that folic acid both lowers tHcy and protects against endothelial injury. As suggested by the authors, screening patients for high tHcy and low platelets has an important public health implication since it will identify a subgroup that will benefit greatly from folic acid treatment.

The importance baseline plasma total cholesterol was also shown in the CSPPT. Patients with cholesterol < 200 mg/dL showed no benefit from folic acid, but those with cholesterol ≥ 200 mg/dL had a 33% reduced risk of first stroke (HR: 0.67 [0.55, 0.82]) (Figure 8).

Several interactions between folic acid treatment and other risk factors were found in the CSPPT, and these are shown in Table 4.

We mentioned above that previous observational studies have found evidence of interactions between raised tHcy and other risk factors in the risk of CVD, including stroke [7,8]; the results from CSPPT are striking confirmation of these interactions, with the strong advantage of it being a primary intervention trial.

This landmark trial has significant public health implications. It means that elderly patients with hypertension whose tHcy is above the 12.5 μmol/L should be given folic acid together with their antihypertensive treatment. Estimates of the impact of folic acid treatment on the period of stroke-free survival have been made from the CSPPT results [116]. Compared with enalapril alone, treatment with enalapril and folic acid predicted a mean lifetime stroke-free survival gain of 1.75 mo, with a maximum gain of up to 12.95 mo. But there was a marked difference according to subgroups: there was a greater gain in stroke-free lifetime with folic acid therapy in younger males, those with lower baseline folate or with higher systolic BP, total cholesterol or fasting glucose. As examples, the authors predicted up to a mean of 5.3 mo of stroke-free life-years could be achieved in male patients with baseline systolic BP higher than 180 mmHg and age younger than 55 y. Up to a mean of 4.2 mo of stroke-free life-years could be gained in patients with glucose higher than 7.0 mmol/L, with total cholesterol higher than 240 mg/dL and age younger than 55 y. There was also a greater gain in those with the CT or TT genotypes of *MTHFR*. Unfortunately, this study did not examine subgroups with different tHcy levels. The authors concluded that, from population health perspective, even this modest gain for individuals may translate into a gain of millions of stroke-free months. This can be illustrated from calculations by Qin et al [112], who found an absolute reduction of 1.3% in first stroke risk in patients with cholesterol > 200 mg/dL treated

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with enalapril and folic acid (2.7%) compared with enalapril-only patients (4.0%), which gives a number needed to treat over 4.5 y of 78. This reduction in risk in hypercholesterolaemia patients treated with enalapril/folic acid could translate into sparing about 2.5 million people in China from stroke over 4.5 years [112].

Lowering tHcy in patients with renal impairment

Patients with end-stage renal disease have a 10-fold greater increased risk of CVD, mainly stroke, than the general population [117]. These patients have markedly raised tHcy [10,34], and the risk of cardiovascular events and mortality is related to the concentration of tHcy [118]. In one study, patients in the top tertile of tHcy had an eightfold greater risk than those in the bottom tertile [119]. The question of whether CVD events can be prevented in patients with end-stage renal disease by lowering tHcy with B vitamins has been studied extensively, but with contradictory results; see reviews [118,120,121]. Likewise, it is controversial whether the progression of renal disease is slowed by tHcy lowering, with the CSPPT showing a beneficial effect of folic acid treatment [122], but other trials have not been positive [120]. Only 3% of participants in the CSPPT were taking antiplatelet drugs, and only 0.8% were taking lipid-lowering drugs [122]. Studies should be done to see whether stratifying for the much more common antiplatelet and lipid-lowering drug use [123,124] in the negative trials in renal disease patients might account for the discrepancy. Although the findings are not consistent, it has been recommended 'While waiting for the results of confirmatory trials, it seems reasonable to consider folic acid with or without vitamin B12 supplementation as appropriate adjunctive therapy in patients with chronic kidney disease' [120].

Vitamin B12

In a wider perspective, we should also consider vitamin B12 status since this has an important influence on tHcy [9]. The median serum B12 in the whole CSPPT cohort was 280 pmol/L (IQR: 231–350) [110], which is within the range of B12 inadequacy [98]. It is therefore possible that addition of B12 to the treatment arm would have led to a bigger fall in tHcy and so to a larger reduction in risk of stroke. Future trials should include B12 along with folic acid. In the meantime, physicians should measure serum B12 in view of the high prevalence of B12 inadequacy in older patients at risk of stroke [35,125]. If B12 is low or low normal, then both vitamins should be given to patients at risk of stroke. This recommendation is especially relevant in countries that have adopted mandatory folic acid fortification of food, since B12 status in these countries is a major determinant of tHcy [126]. It has been recommended that patients with impaired renal function should be given an alternative form of vitamin B12, not cyanocobalamin [121,127], because impaired renal function inhibits the clearance of thiocyanate and so the detoxification of cyanide [128].

Population studies: effect of folic acid fortification

As described above (Figure 2) after the introduction of mandatory food fortification with folic acid by January 1998, the tHcy concentration in the US population fell, with the largest effect in those with higher initial values. The same is true for Canada, which introduced fortification by November 1998 [62]. In the United States, the decline in the median concentration of tHcy in those at particular risk of stroke (those over 60 years old) was a modest 11%. However, there was a much larger fall of 62% in the proportion with tHcy > 13 μmol/L [60].

If raised tHcy is one of the causes of stroke, then a decline in stroke incidence after fortification would be predicted from the large fall in the proportion of the elderly population with high tHcy. A striking report from the Centers for Disease Control and Prevention supports this prediction in that a significant decline in stroke mortality in men and in women occurred both in the United States and Canada after introduction of fortification [129]. The changes in stroke mortality for women in Canada compared with women in England and Wales are shown in Figure 9.

Mortality declines over time in both countries, but in Canada the slope becomes steeper after fortification, whereas in England and Wales, which did not fortify, there is no change in slope. This makes it unlikely that improved treatment was the cause of the changes in the United States and Canada. Confirmation that there had indeed been a decline in the incidence of stroke after fortification, as reflected in the number of hospital admissions, was provided for Canada by Tu et al. [130] and for the United States by the National database [131]. The differences following mandatory fortification are important in public health terms, with about

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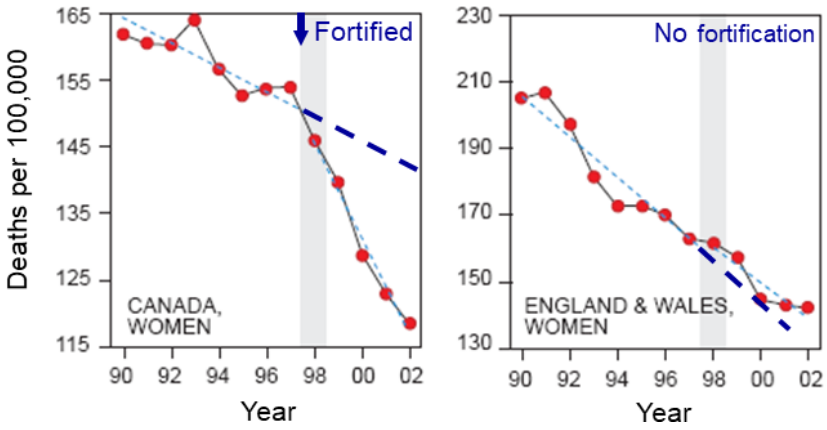

Fig. 9 *Actual and estimated (lines) age-adjusted stroke mortality per 100,000 women in Canada and in England and Wales, 1990-2002. The estimates are based on simple segmented log-linear regression analysis of the observed data. The grey rectangle shows the period when mandatory folic acid fortification was introduced in Canada, and the thick blue dashed lines show the predicted trends after fortification assuming no change. Redrawn from fig. 4 in Yang et al. [129].*

12,900 fewer deaths from stroke per year in the United States (a decline of 2.6%), and about 2,800 fewer in Canada (a decline of 4.4%), than if the trend before fortification had continued [129].

Our overall conclusion from this discussion is that those at risk of stroke due to the presence of other risk factors will benefit from treatment with B vitamins, especially if their tHcy is high. The beneficial effect of B vitamins appears less in those using antiplatelet or lipid-lowering drugs. Measurement of tHcy in subjects at risk of stroke and appropriate treatment is a rational policy with important public health implications. The population benefits of mandatory folic acid fortification for stroke prevention are striking, but have to be considered in relation to any possible harms of fortification [132–135].

Homocysteine and disease prevention – cognitive impairment and dementia

Age-related cognitive impairment and dementia, including AD, are major public health problems, and so, the discovery that raised tHcy was a biomarker for these syndromes (see the excellent historical survey by McCaddon [136]) immediately raised the hope that lowering tHcy might be one avenue to prevent them. Several randomized clinical trials with B vitamins have been done to test this hypothesis. These have been reviewed [44,49,53], and so, we will not review them again, but will summarize our evaluation of the current evidence.

First, based upon Table 3, we list *the conditions that should be fulfilled in any trial of homocysteine-lowering B vitamins in relation to cognition*:

1 Elevated tHcy or suboptimal B vitamin status should be present in the participants so that benefit can occur. No benefit can be expected if the participants already have an adequate B vitamin status. Hence, it is crucial to measure tHcy and/or B vitamins at baseline. It is noteworthy that some trials have not done this, or only done it in a subset of the population.
2 The outcome measured must be sensitive to change over the time period of the trial. Screening tests such as Mini-Mental State Examination (MMSE) have often been used in trials, but these are rarely sensitive enough to detect change over a short time. More specific cognitive tests should be used, and in addition, sensitive physical measurements such as the rate of brain atrophy determined by MRI [137] should be used whenever possible.
3 Participants in the trial should be at risk of cognitive decline or already showing decline, but should not have a diagnosis of dementia. In patients with dementia, it is likely that the degenerative process has proceeded too far for any modification of the disease process to be possible. For example, in the Alzheimer's Disease Co-operative Study trial [138], patients with moderately severe dementia did not benefit from homocysteine-lowering treatment, but those with mild dementia did show some benefit.

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4 The duration of the trial should be long enough to measure clinically relevant change, such as cognitive decline, in the placebo group. This period should be at least 12 mo and preferably 2 y, in particular if conversion to dementia is being assessed. It is noteworthy that many trials do not fulfil the criterion of cognitive decline in the placebo group: for example, in a New Zealand trial, the placebo group had an MMSE score of 29.17 ± 0.16 at baseline and 29.32 ± 1.10 after two y; there was no effect of B vitamin treatment [139]. In the meta-analysis by Clarke et al [140], 76% out of 20,431 participants in the trials did not have baseline measures of cognition and so it was not possible to determine cognitive decline in the placebo group; this fact must cast doubt on the validity of the authors' conclusions.
5 The doses of the vitamins should be sufficient to lower tHcy in the majority of the participants, which means that food-based vitamins will usually not be adequate. Oral doses needed are typically folate 0.4–0.8 mg, B6 10–20 mg and B12 0.5–1 mg.
6 It is crucial that the analyses prespecified in the trial protocol include subgroup analysis in relation to baseline levels of tHcy and/or of the B vitamins, and preferably also change in these markers in response to intervention. This is because no effect of treatment can be expected if the subjects have adequate B vitamin status at baseline (see point 1). A likely finding is that the beneficial effect will be the greater, the higher the baseline tHcy, and that it will be limited to the group that responds to B vitamins with lowering of tHcy.
7 The protocol should specify analyses adjusted, or stratified, according to other factors known to influence cognitive decline, such as age, ApoE genotype and factors such as omega-3 fatty acids, lipid-lowering and antiplatelet drugs [141] that appear to interact specifically with homocysteine or B vitamins.

Relatively few of the many published B vitamin trials or meta-analyses of trials satisfy the above criteria; these are the FACIT trial [142], VITACOG (reviewed in Ref. [49]) and two trials in China [143–146]. Each of these trials reported beneficial effects of B vitamin treatment on cognitive measures.

In the *FACIT trial* in the Netherlands, 819 people (mean age: 60 years) were randomized to daily folic acid (0.8 mg) or placebo for a period of 3 years. The median population baseline tHcy was 12.9 μmol/L, and after 3 years, it was 10.1 μmol/L in the folic acid group, whilst in the placebo group it was 13.4 μmol/L. The cognitive performance of participants was assessed using sensitive tests of different cognitive modalities. In both the placebo and folic acid groups, sensorimotor speed and information processing speed declined, but the decline was slower in the folic acid group. Both groups improved in the memory tests (due to procedural learning effects), but the improvement was greater in the folic acid group. Global cognitive function improved significantly only in the folic acid group. The authors estimated that the effect of folic acid treatment confers performances on certain cognitive tests that are equivalent to those in people several years younger than in the placebo group (Figure 10).

The clinically most relevant results are those on memory. For example, folic acid improved the performance on the 15-word delayed recall subtest to that of an individual 6.9 years younger. There was a significant interaction between baseline tHcy concentration and the effect of folic acid: those with tHcy above 12.9 μmol/L showed a greater improvement in information processing speed than those with tHcy below 12.9. This is in accordance with criterion 6 above.

Several B vitamin trials have been carried out in participants with mild cognitive impairment (MCI). A series of reports from Tianjin in China describe a trial of folic acid (0.4 mg) in 180 participants with

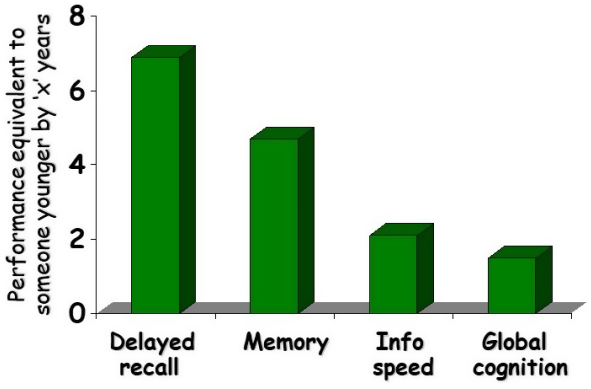

Fig. 10 *Effect of folic acid treatment on several cognitive domains expressed as the equivalent of a person younger by a number of years. Plotted from data in Durga et al. [142].*

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MCI with cognitive assessments at 6 months [143], 12 months [144] and 24 months [145]. Cognition was assessed by the Chinese version of Wechsler Adult Intelligence Scale-Revised. Compared with the placebo group, the folic acid group had improved scores for full-scale IQ and digit span at all three time-points and for block design at 6 mo and information at 12 and 24 months. Folic acid treatment lowered tHcy concentration from the baseline value of 13.65 μmol/L to 9.49, 8.21 and 7.54 μmol/L at 6, 12 and 24 mo, respectively. Another trial from Tianjin had a factorial design and tested the effects of folic acid (0.8 mg), or B12 (25 μg) or a combination of both these B vitamins in 240 patients with MCI [146] for a period of 6 mo. In this cohort, the baseline tHcy was high, with a mean of about 20 μmol/L. No cognitive benefit was seen in the B12-only group, but both the folic acid only group and the combined folic acid/B12 group showed improvements in full-scale IQ, information and digit span. The improvements were greater in the combined folic acid/B12 group than in the folic acid-alone group. Overall, these trials from China show modest cognitive benefits of B vitamin treatment in people with MCI, even over periods as short as 6 mo.

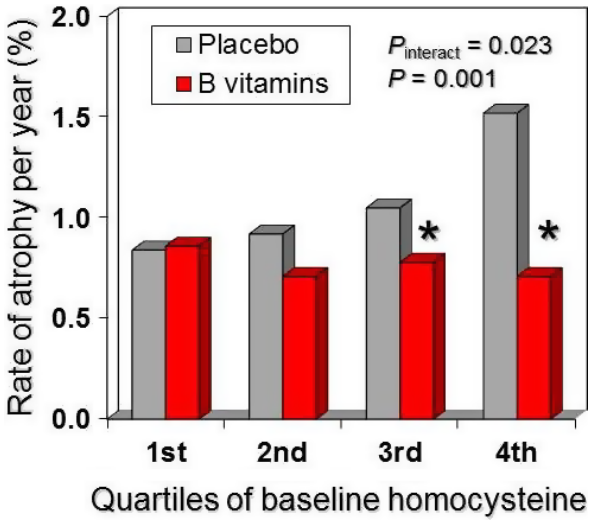

Fig. 11 *Rate of brain atrophy in subjects with mild cognitive impairment in the VITACOG trial. Global brain atrophy rate in relation to quartiles of baseline tHcy concentration. Subjects were treated for 2 years with B vitamins (folic acid, vitamins B6 and B12) or placebo. Plotted from data in Smith et al. [137].*

The primary aim of the *VITACOG trial* in 187 participants with MCI was to see whether lowering tHcy by B vitamin treatment would slow the rate of brain atrophy over 2 years. As we have reviewed this trial previously [49,53,147], we will here focus on aspects related to baseline tHcy as predictor of response. Treatment with folic acid (0.8 mg), B12 (0.5 mg) and B6 (20 mg) in people with MCI slowed global brain atrophy by 29.6%. The effect was greater for participants with higher baseline tHcy concentrations, such that in those in the top quartile (>13.1 μmol/L), the rate of atrophy was slowed by 53% (Figure 11).

Although VITACOG was not powered to detect any effects of B vitamin treatment on cognition, significant effects were in fact found, but only in participants with high baseline tHcy [148]. In the placebo group, only those with baseline tHcy above the median (>11.3 μmol/L) showed cognitive decline, but in this subgroup with high baseline tHcy, B vitamins prevented decline in both episodic and semantic memory and slowed decline in the MMSE score. A significant beneficial effect of B vitamin treatment on the Clinical Dementia Rating (CDR) was observed but was only found in those with tHcy in the top quartile (>13.1 μmol/L).

At baseline, a CDR score = 0 (i.e. 'not impaired') was found in 25.0% of the B vitamin-treated group with baseline tHcy in the top quartile. At follow-up, 58.3% had CDR = 0 (p = 0.039), that is the group experienced a marked clinical improvement. In contrast, those in the high tHcy group taking placebo showed no change in the proportion of CDR = 0 from baseline (24%) to follow-up (28%) (Figure 12).

These results show that, in MCI participants with high baseline tHcy, B vitamin treatment not only slows brain atrophy and cognitive decline but also improves their clinical status.

Further analysis of the MRI scans taken at the start and end of the VITACOG trial has yielded important new information about the relationship between baseline tHcy concentrations and the effect of B vitamin treatment on regional grey matter volume [149]. Application of voxel-based morphometry revealed that in participants with baseline tHcy above the median (11.06 μmol/L), those treated with B vitamins showed much less regional loss of grey matter than the placebo group in multiple regions, including the bilateral

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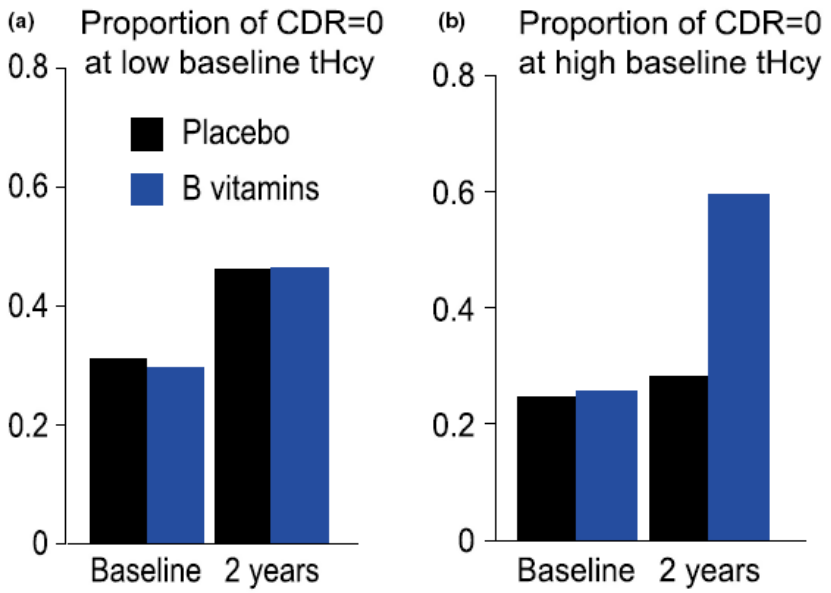

Fig. 12 *Effect of B vitamin treatment on the proportion of participants with a CDR score = 0. 'Low' and 'High' baseline tHcy refer to values below and above the upper quartile (13.1 μmol/L), respectively From [148].*

hippocampus and parahippocampal gyrus, retrosplenial precuneus, lingual and fusiform gyrus, inferior parietal lobule and also the anterior cingulate cortex and piriform cortex and prefrontal areas (Figure 13). These regions are known to be particularly vulnerable in AD, and several are part of the 'parieto-medial temporal pathway' involved in visuospatial learning and spatial long-term memory [150]. The mean rate of atrophy of these particular brain regions over 2 y was reduced from 5.2% in the placebo group to 0.6% in the B vitamin group, a slowing of atrophy by 88%. However, no significant slowing of regional atrophy was found in participants with tHcy below the median. This finding indicates again the importance of studying subgroups.

In relation to the beneficial effect of B vitamins on the CDR score (Figure 11), it is noteworthy that the worsening of the CDR sum-of-boxes score over 2 years was associated with regional atrophy, especially in the medial temporal lobe and that these regions overlapped with those protected by B vitamin treatment (Figure 14).

The results from the VITACOG trial show strong correlations between B vitamin treatment and slowing of regional brain atrophy and of cognitive decline. The authors went further in order to find the mediating pathway between B vitamin administration and the outcomes, using directed acyclic graph analysis to produce the Bayesian network that best explained the data. A very good model fit was obtained that suggests the following causal pathway:

B vitamin treatment (mainly B12)
↓
Lowers tHcy
↓
Slows gray matter atrophy
↓
Slows cognitive decline

The slowing of grey matter atrophy caused the slowing of decline in episodic memory, in semantic memory and in global cognition (MMSE and CDR) [149]. These findings provide strong evidence that lowering elevated tHcy has major cognitive benefits in people with MCI and that tHcy lowering with B vitamins modifies the disease process in MCI [147].

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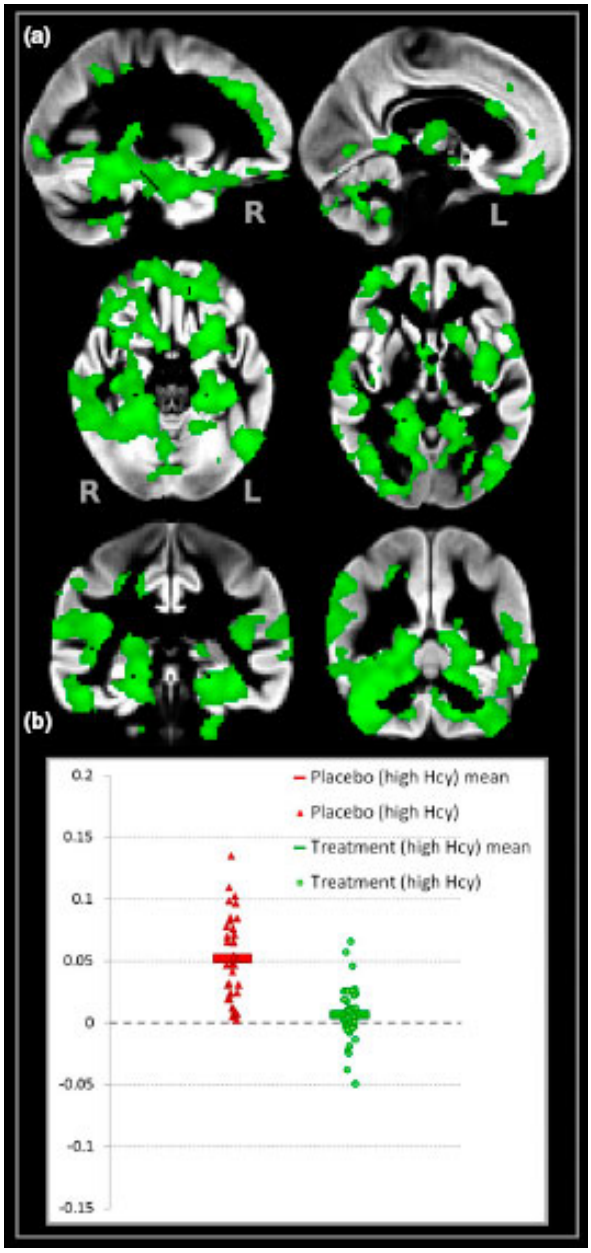

Fig. 13 *(a) Brain regions in green show where B vitamin treatment significantly reduced grey matter loss in participants with baseline tHcy above the median. (b) Percentage of significant grey matter loss over 2 years for those in the placebo and B vitamin groups. From fig. 3 in Douaud et al. [149].*

Interaction between aspirin and B vitamin treatment

Interestingly, as described for CVD, we observed an interaction between aspirin use and the effect of B vitamins on brain atrophy. In those taking aspirin, there was no beneficial effect of B vitamins on brain atrophy. In contrast, other NSAIDs did not appear to modify the effect of B vitamins (Table S2 in reference [137]). NSAIDs also have antiplatelet effect via COX-1 inhibition, but they are weaker than aspirin. Still, if these results are confirmed in other studies, it may suggest that the modifying effect is unique for aspirin and may not be relevant for other antiplatelet drugs. Since prophylactic treatment with aspirin is no longer indicated in older subjects [103], a larger number of elderly with MCI or at risk of dementia will be able to benefit from treatment with B vitamins.

Interaction between homocysteine and omega-3 fatty acid status

A *post hoc* analysis using data from the VITACOG trial revealed an unexpected result: the beneficial effect of B vitamins both on the rate of brain atrophy [151] and on the slowing of cognitive decline [152] only occurred in participants who also had a good plasma omega-3 fatty acid status at baseline. Those in the bottom tertile of omega-3 (<390 μmol/L) showed no beneficial effect of B vitamin treatment on atrophy, whereas for those in the top tertile (> 590 μmol/L), B vitamin treatment slowed atrophy by 40% compared with placebo. In the subgroup with high tHcy (>11.3 μmol/L) and the top tertile of omega-3, B vitamin treatment slowed atrophy by 70% compared with placebo [151]. Significant interactions between B vitamin treatment and omega-3 status were also found for cognitive measures of episodic memory, global cognition and CDR score. For example, at follow-up, in the placebo group, 59% of those in the top tertile of omega-3 had CDR of 0.5 or higher, compared with only 33% in the B vitamin group [152].

The interaction between homocysteine and omega-3 fatty acids also occurs in the other direction. In a retrospective analysis of the samples from the OmegAD trial [153], the beneficial effect of omega-3 fatty acids on cognition in AD was only observed in patients with low baseline tHcy (<11.7 μmol/L) [154]. The findings might explain why several trials of omega-3 fatty acids in cognitive impairment have failed: if the B vitamin status was not adequate, then the omega-3 fatty acids would not have been beneficial.

The idea of an interaction between homocysteine and omega-3 fatty acids was proposed by Selley in

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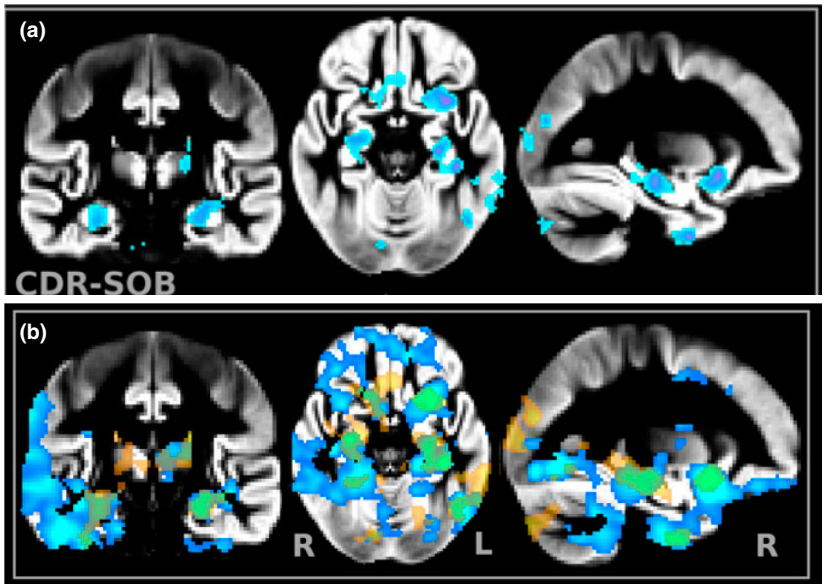

Fig. 14 *Upper part (a) shows blue correlation between grey matter loss and cognitive decline (CDR sum of boxes), mainly in medial temporal areas, entorhinal and piriform cortex. Lower part (b) shows the overlap between these areas (yellow) and the significant protective effect of B vitamins on regional atrophy (blue); overlap appears green. From Fig. S5 and S6 in Douaud et al. [149].*

2007 [155] who postulated that raised tHcy, via its precursor S-adenosylhomocysteine, would inhibit phosphatidylethanolamine N-methyltransferase, so preventing the formation of phosphatidylcholine enriched in omega-3 fatty acids. B vitamins, by lowering tHcy, will therefore facilitate the formation of omega-3 containing phosphatidylcholine, which is crucial for membrane function in the brain. For further discussion, see our review [49].

Further evidence of an interaction has come from a recent trial in Tianjin in which patients with MCI were treated daily for 6 months with either folic acid (0.8mg), or the omega-3 fatty acid docosahexanoic acid (DHA, 0.8mg), or with a combination of both nutrients [156]. Out of 7 cognitive test scores, compared with placebo, 2 were improved by folic acid, 3 were improved by DHA, whilst 5 were improved by a combination of folic and DHA.

Population studies

As described above, the introduction of mandatory folic acid fortification in North America, and the corresponding fall in population tHcy concentrations, was associated with a subsequent decline in the incidence of NTDs and of stroke. Is the same true for cognitive impairment and dementia? This question cannot readily be answered because the end-point is not easily defined. Diagnostic criteria for cognitive impairment and for dementia vary and are difficult to implement uniformly [157]. An example where this has been achieved is the Framingham Heart Study. The 5-years cumulative incidence of dementia has been analysed by the same criteria in over a period of three decades [158]. The incidence was 2.8% in 1986–1991 and 2.0% in 2004–2008, but the decline was only observed in those who had at least a high-school diploma; the same group showed a significant improvement in cardiovascular health. Nevertheless, adjustment for known CVD risk factors (tHcy was not included) did not change the result and the authors concluded that a search should be made for additional explanations. Since tHcy has been measured in the Framingham cohort [159,160], it would be of interest to compare concentrations in the same two periods that dementia was assessed.

It is not just in North America where the incidence of dementia has declined over time: a survey of four European countries, Iceland and the United States found a consistent average decline in incidence rate of 13% per decade between 1988 and 2015 [157]. Since only the United States has implemented mandatory folic acid fortification, this

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cannot be the sole explanation for the decline. On the other hand, the folate status of European populations has probably improved by voluntary fortification and better diets over this period and so the population tHcy might have fallen. Studies are needed to test this hypothesis. The authors of the survey concluded that changes in lifestyle, education and health interventions, such as blood pressure control, all occurred during the past three decades and so it may be impossible to identify one particular factor causing the decline in dementia incidence [157].

An alternative approach is to look at an individual cohort that has been studied over several years. The Hordaland Homocysteine Study has followed more than 18,000 men and women in western Norway since 1992–1993 [9]. Over 2,000 elderly people in the cohort were part of a cognitive substudy and had serum folate and tHcy measured at baseline and 6 years later. At the follow-up, the mean age was 72 years and an episodic memory test was done. High tHcy or low folate at baseline was associated with later memory impairment [161]. In participants whose tHcy declined over the subsequent 6 years, the memory score was higher than for those in whom tHcy increased (Figure 15). The opposite result was found for changes in serum folate.

In context of the prevention of AD, we note that a very comprehensive review and meta-analysis of 143 modifiable risk factors for the disease have concluded '*Notably, homocysteine-lowering treatment seems the most promising intervention for Alzheimer's disease prevention*' [162].

Our conclusion from this brief review of homocysteine-lowering and cognition is that there is robust evidence from a limited number of trials that the disease process in cognitive impairment can be slowed by administration of B vitamins, particularly in people with high tHcy and good omega-3 fatty acids status. It is now crucial that trials are done to see whether a combination of B vitamins and omega-3 fatty acids will slow, or even prevent, conversion from MCI to dementia. If successful, this treatment would have major public health implications.

Can we identify a 'safe' range of tHcy?

As we have described, the range of values for tHcy in a population is quite wide because so many factors influence it. In Table 6 of our review in 2004 [4], we proposed upper reference limits for several groups, depending upon whether or not the population was exposed to folic acid fortification or taking folic acid supplements. We consider that these reference limits are still broadly valid. The question often asked is 'At which concentration of tHcy should action be taken to lower tHcy?' The findings we have summarized in this review lead us to conclude that values of tHcy in the upper normal

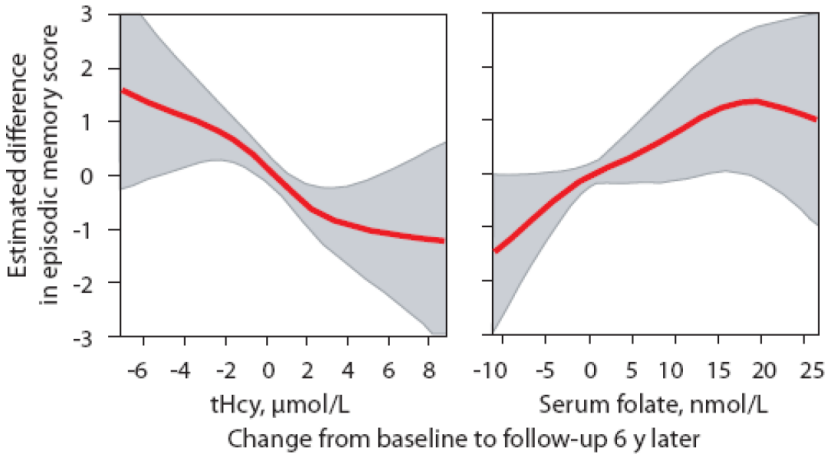

Fig. 15 *Changes in episodic memory (Kendrick Objective Learning Test) over time in relation to plasma tHcy and plasma folate over a 6-years period in a cohort of 1,670 elderly people (mean age: 72 years) in Norway. Solid lines are the estimated concentration–response curves by linear regression; grey areas show the 95% confidence zones, adjusted for sex, baseline analyte, ApoE4, education, CVD and depression. The relationship was significant for tHcy (*p = *0.003) and nearly so for folate (*p = *0.056) (modified from Nurk et al. [161]).*

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range are not totally safe. For major diseases such as stroke or cognitive impairment or dementia, the evidence indicates that values in adults of above about 11 μmol/L may lead to adverse effects and that more certainly values above 13 μmol/L can lead to harm. Thus, we would advise that values in adults of 10 μmol/L or below are probably safe, but that values of 11 μmol/L or above may justify intervention.

Overall conclusion

There are over 100 conditions that are associated with raised concentrations of tHcy (Table 1 and Table S1). The most common associations are with CVD and diseases of the central nervous system. Few other disease biomarkers can match tHcy with so many associations. But a biomarker becomes far more relevant if its modification can prevent development or progression of disease and so improve and maintain health. We have briefly reviewed evidence that five conditions can at least in part be prevented by lowering tHcy: NTDs, impaired childhood cognition, macular degeneration, primary stroke and cognitive impairment in the elderly. Provided that further intervention trials confirm these conclusions, the public health implications of the findings are considerable. It is critical that future trials are designed so that they target groups that are likely to benefit. The fact that the treatment is safe, cheap and effective should make it readily adopted worldwide.

Conflict of interest

Dr Smith reported being named as inventor on 4 patents held by the University of Oxford on the use of B vitamins to treat Alzheimer disease or mild cognitive impairment (US6008221, US6127370, PCT/GB2010/051557 and WO 2015/140545). Dr Smith is on the scientific advisory board for Elysium Health, NY, and a consultant for Aprofol. Dr Refsum reported being named as inventor on 2 patents held by the University of Oxford on the use of B vitamins to treat mild cognitive impairment (PCT/GB2010/051557 and WO 2015/140545).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Plasma total homocysteine as a disease biomarker.

Table S2. Lowering homocysteine and disease prevention.

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